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Association Between *Leptotrichia Amnionii* and *Atopobium Vaginae* as A Risk Factor For Miscarriage States in A Specimens of Iraqi Women

Ghufran TH. Akram*, Nuha J. Kandala

Department of Biotechnology, College of science, University of Baghdad, Baghdad, Iraq

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Abstract

Miscarriage is one of the fundamental complications occurring in pregnant women. Many fastidious and uncultivated bacterial species are related to miscarriage and have a significant role in the infection. The association between inverse pregnancy outcomes and infections of abnormal bacteria has been rarely investigated. Therefore, this study aimed to determine *Leptotrichia amnionii* and *Atopobium vaginae* as a risk factor for miscarriage states. A total of 80 cervical swabs and blood samples were obtained from females (34 non-pregnant with recurrent spontaneous miscarriage, 11 pregnant who had previous miscarriage, and 35 without miscarriage as control) who were referred to a hospital in Baghdad city. The relationship between demographic characteristics and miscarriage groups indicates a positive association between the age of 30 years and >45 years and miscarriage states, while no significant association ($p>0.05$) was found for the other parameters compared with the control. Also, no association was observed between the levels of TORCH IgM, antiphospholipid (APL) IgM, and anticardiolipidin (ACL) IgM antibodies and spontaneous miscarriage. The findings of RT-PCR detection revealed that, out of 45 samples of recurrent spontaneous miscarriage, 17 were detected with high-risk of *L. amnionia* compared with two samples from the control group. Also, 7 samples of recurrent spontaneous miscarriage were shown to be infected with *A. vaginae* compared with five samples from the control group. The adjusted odd ratios (ORs) of *L. amnionii* for spontaneous miscarriage and pregnancy states were 7.89 (CI 95%, 1.60 - 39.00) and 9.43 (CI 95%, 1.43 - 61.9), respectively, with highly significant difference between the study groups compared with the control. While no significant result was recorded between the adjusted ORs of *A. vaginae* for spontaneous miscarriage and pregnancy states, which were 1.23 (CI 95%, 0.416 - 3.67) and 0.636 (CI 95%, 0.083 - 4.88), respectively, compared with the control.

Keywords: spontaneous miscarriage, *Leptotrichia amnionii*, *Atopobium vaginae* immunological factors

العلاقة بين بكتريا *leptotrichia amnionii* و *Atopobium vaginae* كعامل خطورة في

حالات الاجهاض في عينة من النساء العراقيات

غفران ثائر اكرم , نهى جوزيف قندلا

قسم التقنيات الاحيائية ، كلية العلوم ، جامعة بغداد ، بغداد ، العراق

الخلاصة

الإجهاض هو أحد المضاعفات الأساسية التي تحدث عند النساء الحوامل. ترتبط العديد من أنواع البكتيريا التي تحتاج إلى متطلبات تغذية معقدة ووصوعية في الاستزراع بالإجهاض والتي يكون لها دور مهم في العدوى و نتيجة ندرة الأبحاث التي تتعلق في دراسة العلاقة بين نتائج الحمل العكسي والالتهابات البكتيرية غير الطبيعية. لذا هدفت هذه الدراسة إلى تحديد بكتيريا *leptotrichia amnionii* و *Atopobium vaginae* كعوامل خطيرة في حالات الإجهاض. تم جمع مسحات من عنق الرحم وعينات دم من 80 أنثى (34 عينة من حالات الإجهاض التلقائي المتكرر في النساء غير الحوامل ، و 11 من النساء الحوامل لديهم حالات إجهاض سابقة و 35 امرأة دون إجهاض (غير حوامل) كمجموعة سيطرة) تم جمعها من مستشفى في مدينة بغداد. تشير العلاقة بين الخصائص الديموغرافية ومجموعات الإجهاض إلى وجود علاقة إيجابية بين الأعمار التي تراوحت بين 30 عامًا أو أكبر و حالات الإجهاض ، بينما لا توجد علاقة معنوية بين المعايير الأخرى التي تضمنتها الدراسة مقارنة بالسيطرة. وكذلك اظهرت نتيجة سلبية لاختبارات للأجسام المضادة الـ IgM TORCH ، والأجسام المضادة IgM للفوسفوليبيد (APL) والأجسام المضادة IgM للكارديوليبيدين (ACL)، وعدم الترابط مع مجاميع الإجهاض التلقائي. وبينت نتائج الكشف باستعمال RT-PCR أنه من بين 80 عينة ، اظهرت 17 عينة تواجد *L. aminonia* . كعامل خطيرة عالية مقارنة مع عينتين من مجموعة السيطرة، في حين ظهرت *A. vaginae* في 7 عينات من مجموعات الإجهاض التلقائي المتكرر مقارنة مع خمس عينات من مجموعة السيطرة. بينما اظهرت *L. amnionii* ترابطا معنويا كبيرا اذ بلغت قيم ORS 7.89 (95% CI -1.43-61.9) و 9.43 (95% CI -1.43-61.9) للإجهاض التلقائي والحمل مقارنة مع مجموعة السيطرة ، في حين لم يكون هناك ترابط معنوي في نتائج الـ *A. vaginae* عندما بلغت قيم ORS 1.23 (95% CI -3.67-0.416) و 0.636 (95% CI -0.083-4.88) للإجهاض التلقائي والحمل.

Introduction

Miscarriage refers to pregnancy loss at less than 20 weeks of gestation; it is the most common complication of pregnancy. Several etiologies factors such as anatomic, genetic, endocrine, hematological and immunological factors have been reported to associate with this destructive complication of pregnancy [1, 2]. Since the word “abortion” is associated with elective termination, the term “miscarriage” is synonymous and often used with patients [3]. First-trimester miscarriage refers to the early stage in the course of a miscarriage when intact gestational sacs are still present within the uterine cavity, blighted ovum; missed, delayed, silent miscarriage, or early fetal demise. The diagnosis of early fetal demise is based either on the absence of cardiac activity in a visible embryo or on the absence of an embryo within a gestational sac [4]. Second-trimester pregnancy loss is defined as pregnancy loss after the 14th week and before the 24th week of gestation. However, it has been associated with infection, cervical insufficiency, uterine malformations, gene polymorphisms, fetal and placental anomalies, and genetic and acquired thrombophilia [5]. Recurrent spontaneous miscarriage (RSM) is described as the occurrence of 3 or more consecutive spontaneous miscarriages, in a series of conditions that lead to or could terminate in reproductive failure [6].

Recurrent spontaneous miscarriage is multifactorial, so that approximately half of all cases will remain unexplained. At present, there exist a small number of accepted etiologies for RSM. These include uncontrolled diabetes mellitus, untreated hypothyroidism, parental chromosomal abnormalities, anti-phospholipid antibody syndrome (APS), and certain uterine anatomic abnormalities. Other probable or possible etiologies include heritable and/or acquired thrombophilia, immunologic abnormalities, additional endocrine disorders, environmental factors, and infection [7, 8]. The correlation between the adverse outcomes of pregnancy and host infections was poorly examined, whereas intrauterine infections that occur in early and late pregnancy leading to a spontaneous miscarriage and spontaneous preterm birth were recorded [9]. Since the main route of placenta and fetus infection is from the vagina and cervix, most studies have attempted to find a link between miscarriage and abnormal bacterial flora in the lower genital tract [10]. However, bacterial vaginosis was reported to be causative for second-trimester miscarriage or premature delivery in the

first trimester of pregnancy [11], although bacterial vaginosis-related bacteria and their toxins can cross the placenta and cause fetal complications [12].

Atopobium vaginaean bacteria are anaerobic, Gram-positive, non-mobile, sporogenous and non-capsulated, measuring approximately 0.6-0.9 μm and forming small clear colonies similar to small pinheads in Columbia blood agar cultures at 37°C [13]. In addition to the highlighted position in BV, *A. vaginae* infection and a higher risk of premature birth was associated [14]. *Leptotrichia* species are fastidious gram-negative, non-motile, long, fusiform rods, non-spore forming, anaerobic bacteria that exist as part of microorganisms of the human oral cavity and female genitalia [15]. A *Leptotrichia* sp. was first reported in 2002, when Shukla et al. [16] described *L. amnionii* as a novel causative species of infection in the female urogenital tract. Also, *L. amnionii* was isolated from the cervix of a patient with a premature rupture of the fetus membranes [17]. PCR analysis has been useful in detecting infections in patients with premature labour, intact membranes, and intra-amniotic infections compared to normal microbial cultures [18]. However, epidemiological data on the potential association between cervical microbiota and spontaneous miscarriage has been rarely reported. The objective of this study was to determine the cervical bacteria in different miscarriage samples using qPCR technique and investigate the correlation between spontaneous miscarriage and cervical bacteria in a sample of Iraqi women.

Materials and Methods

The Subjects

All samples required in this study were collected from women (age ranged 18-51 years) attended as outpatients of gynecology department of the Medical City Teaching Hospital. Patient's samples were selected according to questionnaire information, history of spontaneous abortion, and number of abortions (and other categories). The study included 80 females, 45 suffering a recurrent spontaneous miscarriage and 35 normal). This study was approved by the Ethics and Research Committee of the hospital under the supervision of the consultant, while approval for the sampling was obtained from patients and controls.

Specimen collection

Two types of samples were collected from women; first, 80 cervical swabs from the endocervical region after inserting a sterile speculum into the vagina. Second, blood samples (5 of venous blood) which were then transferred into polyethylene tubes, allowed to clot, and centrifuged at 3000 rpm for 10 minutes. The obtained clear serum was frozen at -20 °C until used for further immunological assays.

Immunochemistry detection of antibodies

In order to detect some microorganisms that maybe related to miscarriage in women, TORCH is a rapid test kit used in this study for the qualitative detection of TOX-IgM, RU-IgM, CMV-IgM, HSV-2-IgM, and HSV-1-IgM antibodies in human serum. The procedure followed the manufacturer's instructions (TORCH rapid test kit, Weifang Kanghua Biotech\China) [19]

Determination of Anticardiolipin IgM and Antiphospholipid IgM antibodies

The detection of antiphospholipid (APL) IgM and anticardiolipin(ACL) IgM antibodies in the serum by was conducted using ELISA Kit, following the manufacturer's instructions (Antiphospholipid and Anticardiolipin antibodies ELISA Kit, Cortez \ USA) [20, 21].

Extraction of DNA

Total DNA (genomic and bacterial DNA) was isolated from cervical swab samples for molecular studies following the standard protocol for extraction. Genomic DNA isolation was achieved by using a DNA-sorb-A nucleic acid extraction kit. Nanodrop instrument (Nas-99/China) was used to determine DNA concentration and purity. The integrity of DNA was estimated by agarose gel electrophoresis.

Real -Time Polymerase Chain Reaction For the qualitative detection of *Leptotrichia amnionii* and *Atopobium vaginae*

Real Time- for *L. amnionii* and *A. Vaginae* were conducted by using 16S rRNA primers (Table-1). The reaction mixture consisted of 5 μl DNA template, 0.8 μl of each primer, 1.25 μl CXR dye, 12.5 μl of Bright Green Express 1X qPCR Master mix, and 4.65 μl Nuclease-free water, in a total volume of 25 μl . The template DNA was amplified for 40 cycles of denaturation for 1 minute at 95°C, annealing of primers at 63°C for *A. vaginae* and 64°C for *L. amnionii* for 1 minute, and extension at 72°C for 1 minute. Fluorescent data were acquired during each extension phase [22].

Table 1- The Oligonucleotide Sequences of Primers for RT-qPCR Detection of *L. amnionii* and *A. vaginae*

The name of gene	Primer sequence 5' → 3'	size product
<i>16S rRNA/A. vaginae</i>	F-GCAGGGACGAGGCCGCAA R-GTGTTTCCACTGCTTCACCTAA	558
<i>16S rRNA for L. amnionii</i>	F-CAATTCTGTGTGTGTGAAGAAG R-ACAGTTTTGTAGGCAAGCCTAT	229

Statistical Analysis

The SPSS software (V 20.0) was utilized to estimate the effects of different factors on the study parameters. The correlation amongst the parameters was estimated using Pearson chi-squared (R) to test for significant differences at $p < 0.05$. Chi-squared and ANOVA tests were used to assess the differences in the categorical and continuous variables, respectively, among miscarriage patients.

Results and Discussion

A total of 80 specimens of endocervical cytobrush and blood were collected from both patients and control groups by a specialist physician at the gynecology department of the Medical City Teaching Hospital. These samples were divided into two groups. First, the patients group consisting of 45 women suffering a spontaneous miscarriage, divided into non-pregnant and pregnant women with previous spontaneous miscarriage. Second, the control group consisting of 35 healthy females who never had a miscarriage.

The effects of different factors on the study parameters

Dependent on the questionnaire form, seven parameters were used to classify the specimens according to the age, body-mass index (kg/m²), menopausal status, education level, number of miscarriages, number of children, and presence of other diseases. The findings of this study showed significant differences between study groups. The age (mean \pm standard deviation) value of the spontaneous miscarriage group was 31.03 ± 7.71 while that for pregnancy group was 26.18 ± 4.35 years. According to a study by **Gracia et al.** [23], the age is considered as an independent risk factor for recurrent miscarriages. Women between the ages of 25 and 30 years had the lowest risk of spontaneous abortion, while the highest risk with an extreme level. Another study by **Shawky et al.** [24] reported that the number of miscarriage women at the age higher than 30 or the end of the 30s was the double compared to women without miscarriages.

In addition, the present study found no significant differences in the parameters of body mass index, education level, menopausal status, number of miscarriage, and presence of other diseases (Table-2).

Table 2- General parameters of the study according to miscarriage status: spontaneous miscarriage group, pregnancy group (had a history of miscarriage) and control (never had a miscarriage).

	Total no.	Control	Spontaneous Miscarriage	Pregnancy	P-Value‡
Age (years) Mean \pm SD					
Age	80	33.17 \pm 7.78	31.03 \pm 7.71	26.18 \pm 4.35	0.027 *
Body-mass Index(kg/m²) (Mean \pm SD)					
index(kg/m ²)	80	28.08 \pm 5.81	26.97 \pm 4.02	25.12 \pm 2.35	0.191 ^{N.S}
Education level N (% of total)					
Middle school	40	16 (20.0%)	18 (22.5%)	6 (7.5%)	0.732 ^{N.S}
High school	27	14 (17.5 %)	9(11.3%)	4 (5.0%)	

University	13	5 (6.3 %)	7 (8.8%)	1 (1.3%)	
Menopausal status N (% of total)					
Premenopausal	74	32 (40.0%)	31 (38.8%)	11 (13.8%)	0.596 ^{N.S}
Postmenopausal	6	3 (3.8%)	3 (3.8%)	0 (0.0%)	
Number of children N (% of total)					
1 or no	31	6 (7.5%)	18 (22.5%)	7 (8.8%)	0.003 ^{**}
2	13	5 (6.3%)	7 (8.8%)	1 (1.3%)	
3 or more	36	24 (30.0%)	9 (11.3%)	3 (3.8)	
No. of miscarriage N (% of total)					
2 or less	29	0	23 (51.1%)	6 (13.3%)	0.724 ^{N.S}
3	10	0	7 (15.6%)	3 (6.7%)	
4 or more	6	0	4 (8.9%)	2(4.4%)	
Other diseases					
Diabetes mellitus	80	0	1 (1.3%)	11 (1.3%)	0.264 ^{N.S}
Infection		4 (5.0%)	0 (0.0%)	0 (0.0%)	
Hypothyroidism		2 (2.5%)	0 (0.0%)	0 (0.0%)	
Heart disease		1 (1.3%)	0 (0.0%)	0 (0.0%)	

Non-significant (NS),*significant $P < 0.05$

Detection of TORCH by Immunochromatography Test

The findings showed that all the 45 specimens of females with recurrent spontaneous miscarriage were negative for TORCH test (TOX-IgM, RU-IgM, CMV-IgM, HSV-2-IgM, and HSV-1-IgM). These findings confirmed that these cases did not include congenital and perinatal infections, as previously indicated by Suryawanshi and co-workers [25].

In spite of that, other studies revealed the effects of pathogens including toxoplasmosis, rubella, cytomegalovirus and herpes simplex. Thus, the role of TORCH complex in determining abortion remains controversial [26], as little information is available about the impact of TORCH infections on the outcome of pregnancy and the causes of miscarriage [27].

Determination of Anticardiolipin IgM and Antiphospholipid IgM antibodies

Out of 45 miscarriage females included, 34 were with spontaneous miscarriage and 11 were under pregnancy. All the specimens were in the normal range ACL (<7 U/ml) APL (<12U/ml) IgM antibodies, indicative that both antibody types do not have a role in miscarriage cases. A previous study [28] indicated that females with antiphospholipid syndrome also have a higher risk of miscarriages, with recurrent miscarriages in some cases becoming an indicator for antiphospholipid testing. Therefore, the possibility of miscarriage due to these factors was excluded in this.

Molecular Detection

Extraction of DNA

Total DNA (genomic and bacterial DNA) was extracted from cervical swab specimens for molecular studies. The results showed that the purity ranged 1.7-2.0 and the concentration ranged 20-50 $\mu\text{g}/\mu\text{L}$ (Figure-1).

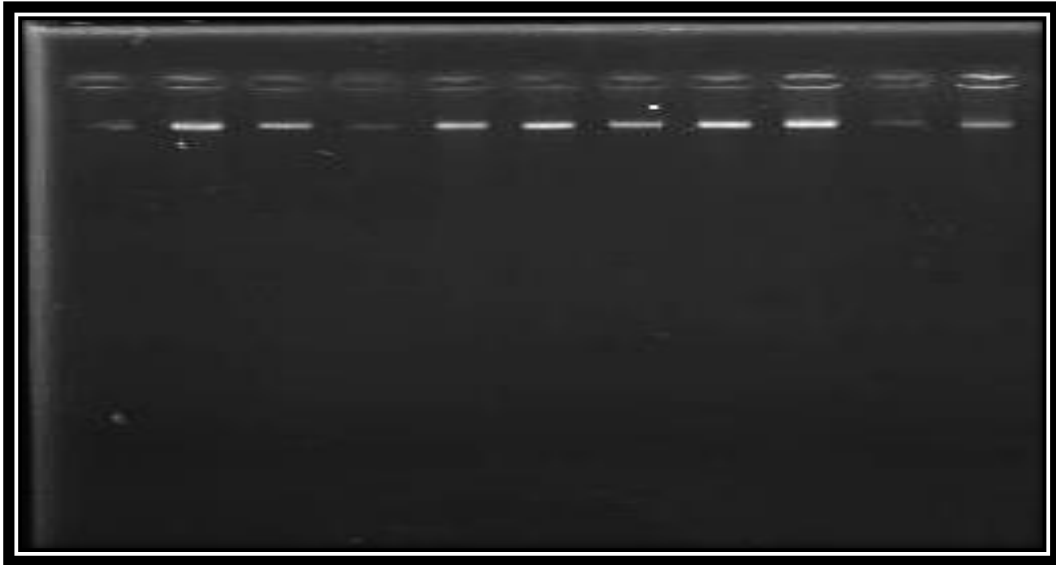
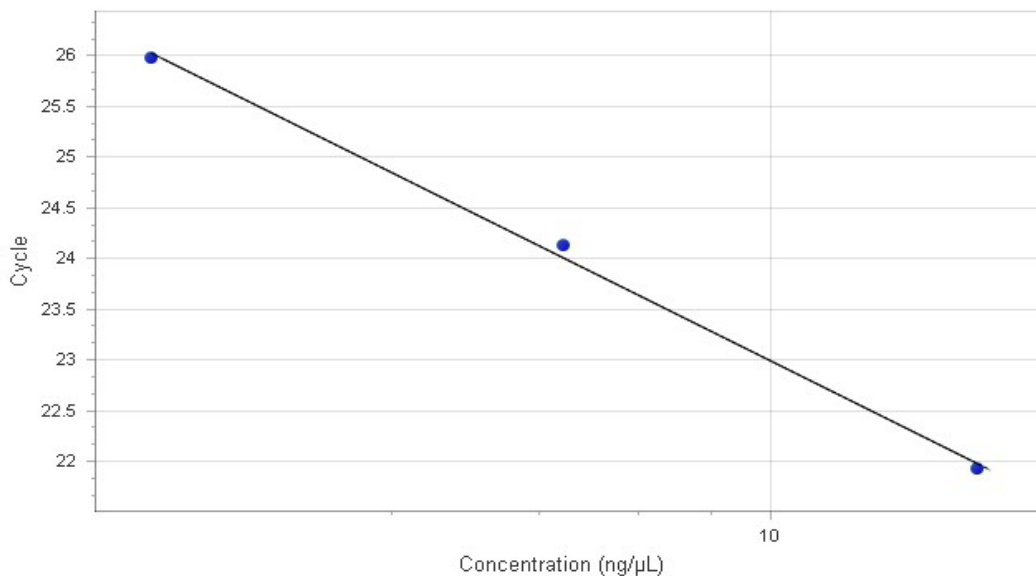


Figure 1- Total DNA on 1% agarose gel at 90 volts for 60 min followed by ethidium bromide staining for 15 min and UV light visualization. DNA samples were extracted from endocervical cytobrush specimens.

Detection of *A.vaginae* Using Real -Time PCR Technique

According to the standard curve of primer efficiency, the positive result of *A.vaginae* is ranging from 13 to 27 Ct (cycle threshold), as shown in Figure-2. The results showed that out of a total of 80 specimens, including 34 of spontaneous miscarriages, 11 pregnancies, and 35 controls, only 6, 1, and 5 specimens, respectively, were positive for *A. vaginae*. The green fluorophore channel was used to detect the 16S rRNA gene of *A. vaginae* in the samples (Figure-3), and the melting curves are shown in (Figure-4). The findings agree with a study by **Harper et al.** [29] who noticed no evidence of a synergistic interaction for the coexistence of vaginosis bacteria and periodontal disease on preterm birth. While the study of **Knoester et al.** [30] suggested an association between maternal bacteremia with *A. vaginae* and fetal death following Chorionic Villus Sampling. Another local study by **Mahdey et al.** [31] specified a high risk of abortion with the infection by *Megasphaera* and *Atobopium*. The study applied qPCR for 16S rRNA gene of bacterial vaginosis as a target with specific and sensitive diagnosis in 100 swabs of Iraqi women with vaginitis.



Standard Curve: Equation: $y = -3.36 x + 26.35$, **Efficiency:** 0.98, **R²:** 0.9975

Figure 2- Standard Curve of Primer Efficiency for *A.vaginae*

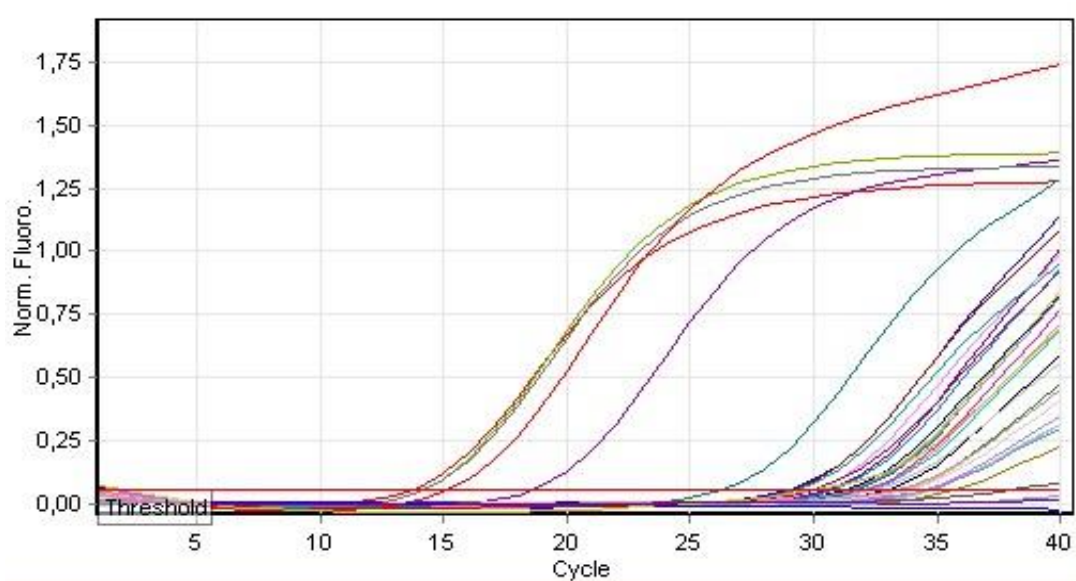


Figure 3- Amplification Curves of *A.vaginae* from Target DNA in Specimens of Spontaneous Miscarriage Group.

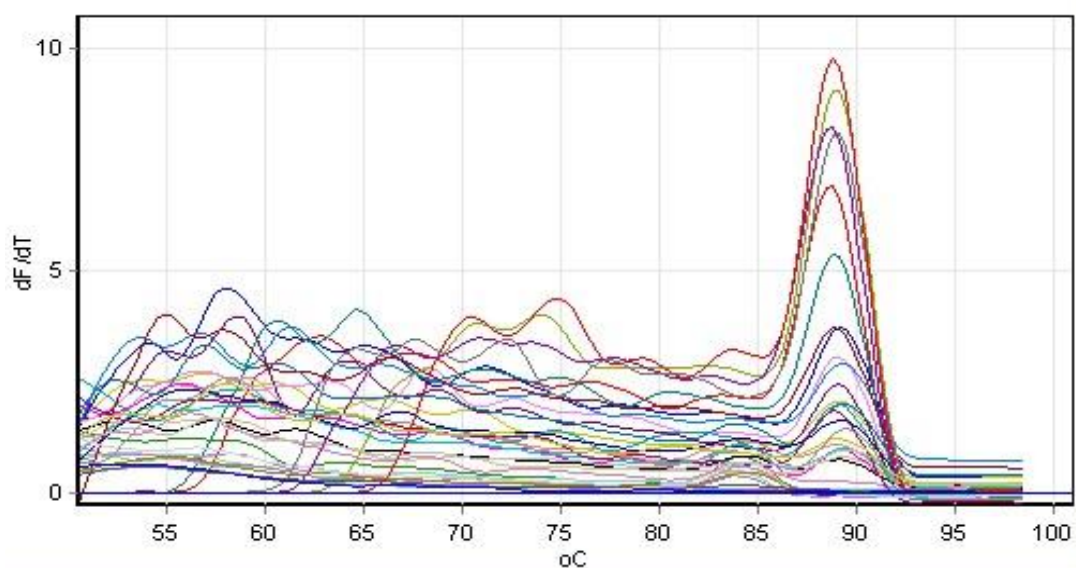


Figure 4- Melting Curves of *A.vaginae* From Spontaneous Miscarriage Group Specimens.

However, the products of RT-PCR were analyzed by gel electrophoresis to detect the product length for *16S rRNA* gene. The results revealed that the molecular size of *A. vaginae* after staining with ethidium bromide and visualization under UV light was of 558 bp, as shown in (Figure -5). Also, the product length was used as an internal control for the gene [32].

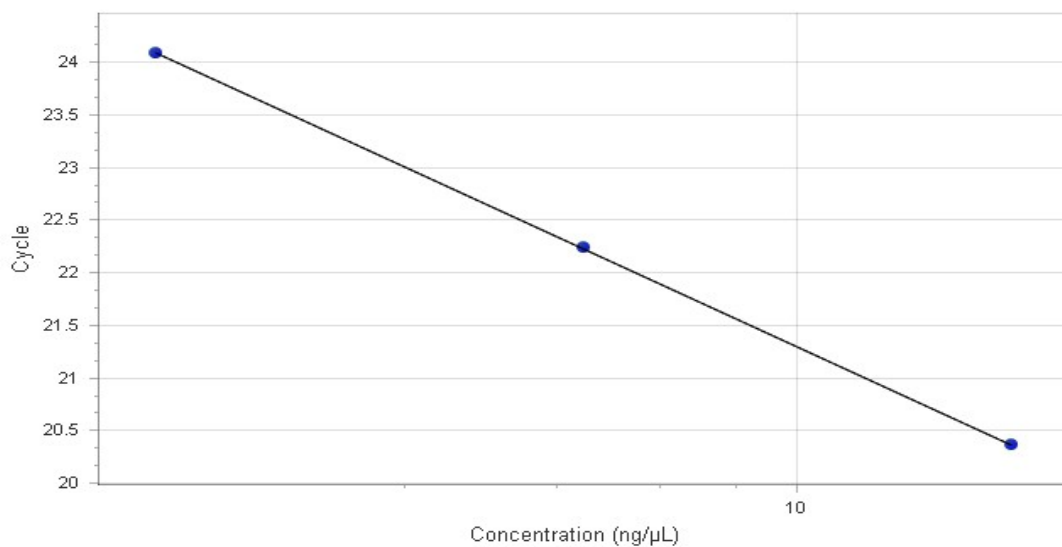


Figure 5- 16S rRNA gene RT-PCR product of 558bp molecular size. Electrophoresis was applied as 2% agarose at 90 volts for 75min., followed by ethidium bromide staining for 20 min., then visualized by gel documentation system. L: DNA ladder (100-1000 bp); Lane (1): (ct value -ve), Lane (2): (ct value 33), Lane (3): (ct value 32), Lane (4): (ct value 33), Lane (5): (ct value 32), Lane (6): (ct value 14), Lane (7): (ct value 33), Lane (8): C (ct value -ve), Lane (9): (ct value 33), NC: Negative control.

Qualitative Detection of *L.amnionii* Using Real Time - PCR

The positive result of *L.amnionii* has a range of 14-26, which is represented in Ct according to the standard curve of primer efficiency, as shown in (Figure -6).

The results of RT-qPCR detection showed that, out of 34 specimens of spontaneous miscarriage, 11 pregnancies, and 35 controls, only 11, 4, and 2 specimens, respectively, were positive for *L. amnionii*.



Standard Curve: Equation: $y = -3.09x + 24.39$, Efficiency: 1.10, $R^2: 1.0000$

Figure 6- Standard Curve for Primer Efficiency of *Leptotrichia amnionii*.

The green fluorophore channel was used to detect the 16S rRNA gene of *L. amnionii* in the specimens (Figure -7 and Figure -8), while the melting curves are shown in (Figure -9). The findings agree with the study of Shukla *et al.* [16] who reported that a novel bacterium was isolated from the amniotic fluid of women who experienced intrauterine fetal death during the second trimester of pregnancy.

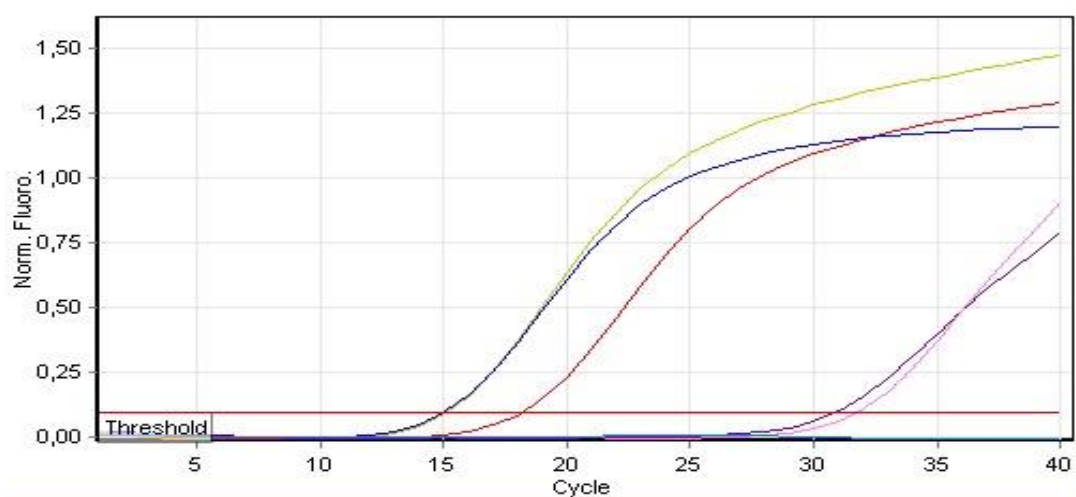


Figure 7- Amplification Curves of *L.amnionii* From Target DNA in a Samples of Pregnancy Group.

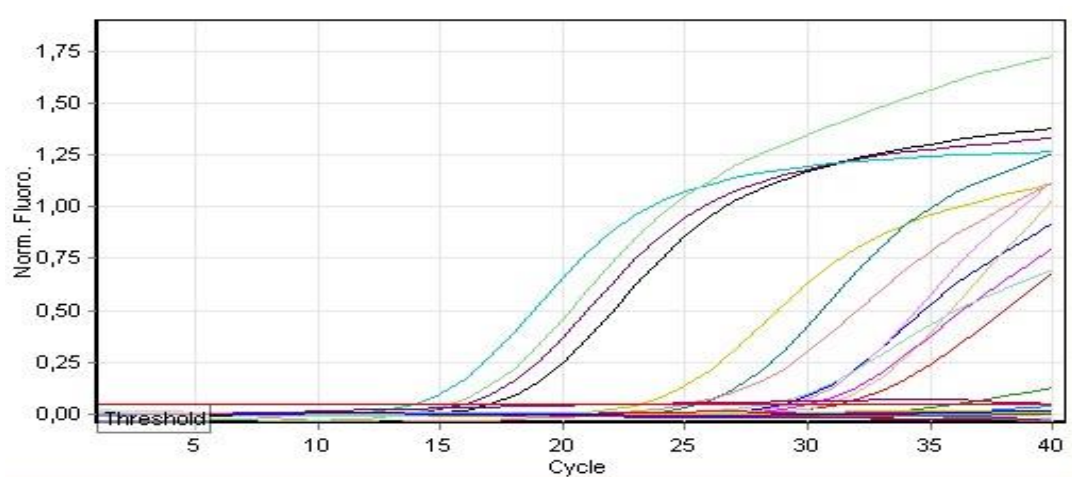


Figure 8- Amplification Curves of *L.amnionii* From Target DNA in a Samples of Miscarriage Group.

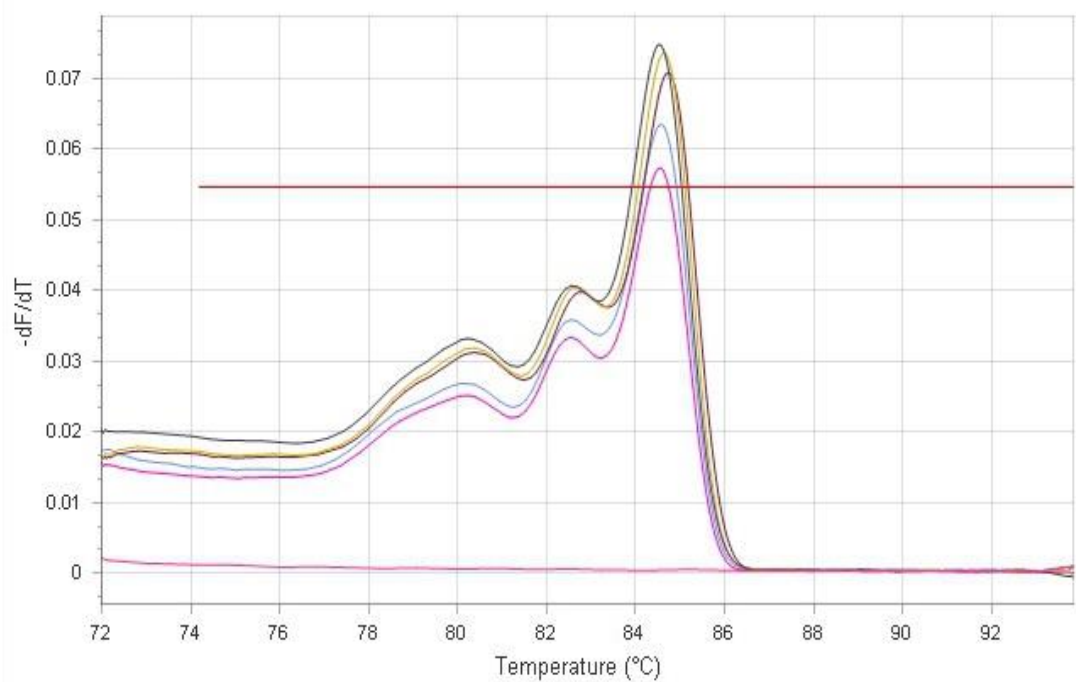


Figure 9- Melting Curves of *L.amnionii* in Spontaneous Miscarriage Group specimens.

However, the products of RT-PCR were analyzed by gel electrophoresis to detect the product length for *16S rRNA* gene of *L.amnionii* after staining with ethidium bromide and visualization under UV light, which had a molecular size of 229bp as shown in (Figure -10). Also, the product length was used as an internal control for the gene [32].

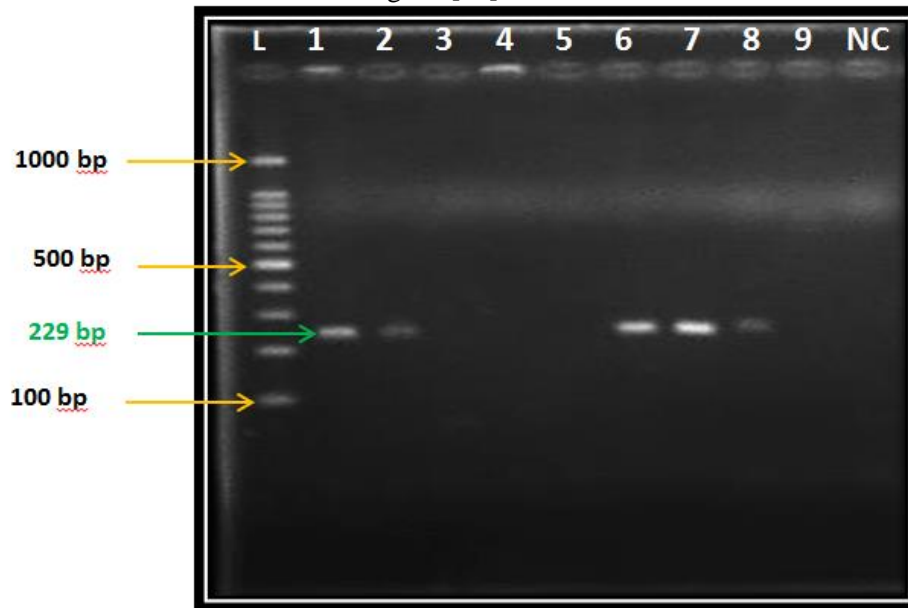


Figure 10-*16S rRNA* gene RT-PCR product of 229 bp molecular size. Electrophoresis was applied as 2% agarose at 90 volt for 75min., followed by ethidium bromide staining for 20 min., then visualized by gel documentation system. L: DNA ladder (100-1000 bp);

Lane (1): 15A (ct value 25), Lane (2): 16A (ct value 26), Lane (3): 13A (ct value -ve), Lane (4): 14A (ct value 35), Lane (5): 21C (ct value -ve), Lane (6): 18A (ct value 19), Lane (7): 27A (ct value 16), Lane (8): 17A (ct value 28), Lane (9): 22C (ct value 33), NC: Negative control.

Association between *A.vaginae*, *L.amnionii*, and Spontaneous Miscarriage

The results of this study showed no significant associations ($P= 0.778$) between infections with *A.vaginae* and spontaneous miscarriage, according to the results of RT-PCR for *16S rRNA* gene that were positive in 6/34 (17.6 %) of the spontaneous miscarriage group and 1/11 (9.1%) of the pregnancy group, compared with 5/35(14.3%) in the control group. These results disagree with other studies which showed that the infection with *A.vaginae* was strongly associated with spontaneous miscarriage [33, 34].

Moreover, the results of the present study showed significant associations ($P= 0.011$) between the infection with *L.amnionii* and spontaneous miscarriage, according to the results of RT-PCR for *16S rRNA* gene that were positive in 11/34 (32.4%) of spontaneous miscarriage group, 4/11 (36.4%) of pregnancy group, and 2/35(5.7%) of control group (Figure-11). These results agree with those of earlier studies which showed that the infection with *L.amnionii* is associated with spontaneous miscarriage [35, 36].

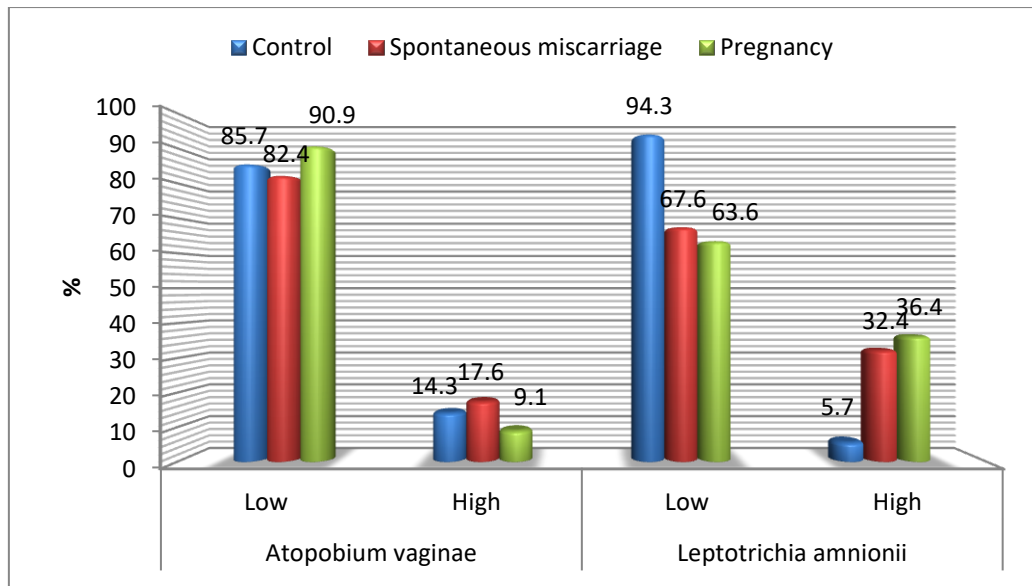


Figure 11- The Association patterns between *A. vaginae* and *L. amnionii* among Control, Spontaneous Miscarriage and Pregnancy Groups (Low: Negative results, High: positive results).

Table 3- The Association of Selected Microbes among Control, Spontaneous Miscarriage, and Pregnancy Groups, Reported as the Odds Ratio (OR) and 95% CI.

Bacteria	Group	Control		Spontaneous miscarriage		Pregnancy		Univariate (ORs, CI 95%)	
		N	%	N	%	N	%	Spontaneous miscarriage	Pregnancy
<i>Atopobium vaginae</i>	Low	30	85.7	28	82.4	10	90.9	1 (ref.)	1 (ref.)
	High	5	14.3	6	17.6	1	9.1	1.23 (0.416 - 3.67)	0.636 (0.083 - 4.88)
Chi square (P-value)		0.778 ^{N.S}				0.703 ^{N.S}		0.655 ^{N.S}	
<i>Leptotrichia amnionii</i>	Low	33	94.3	23	67.6	7	63.6	1 (ref.)	1 (ref.)
	High	2	5.7	11	32.4	4	36.4	7.89 (1.60 - 39.00)	9.43 (1.43 - 61.9)
Chi square (P-value)		0.011*				0.005**		0.008**	

¥ Chi-squared test of association was used, N.S: not significant ($P > 0.05$), ORs, 95% Ci: Odd ratio and confidence interval (95%). Highly significant**, significant*.

The results in Table -3 show no significant association between *A. vaginae* and spontaneous miscarriage and pregnancy groups compared with the control group ($p = 0.778$). The adjusted ORs of *A. vaginae* for spontaneous miscarriage and pregnancy were 1.23 (CI 95%, 0.416 - 3.67) and 0.636 (CI 95%, 0.083 - 4.88) with p-value of 0.703 and 0.655, respectively. These results do not support the assumption that spontaneous miscarriage also associates with the involvement of *A. vaginae*. Infection with *A. vaginae* was reported to possibly cause maternal sepsis and spontaneous miscarriage [30].

Meanwhile, the results of *L. amnionii* show significant association with spontaneous miscarriage and pregnancy groups compared with the control group ($p = 0.011$). The adjusted ORs of *L. amnionii* for spontaneous miscarriage and pregnancy were 7.89 (CI 95%, 1.60 - 39.00) and 9.43 (CI 95%, 1.43 - 61.9), showing highly significant association with p-value of 0.005 and 0.008, respectively, compared with the control group. These results support the claim that a previous Norwegian case study of *L. amnionii* was the first to differentiate patients with chorioamnionitis *L. amnionii* from a renal abscess in spontaneous miscarriage [17]. Cumulatively, these results suggest a prospective look into the association between the microbiota, including *L. amnionii*, found in the cervix with spontaneous miscarriage.

In conclusion, fetal loss is considered high in women in the age range of the 30s. This is due to that recurrent miscarriage and other complications in pregnant women might share underlying causes, which could be biological conditions. Infection with *L. amnionii* showed a strong association with spontaneous miscarriage and pregnancy groups, which found highly significant differences compared with the results of *A. vaginae* in the two groups.

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